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ORIGINAL ARTICLE

Current status of hepatic glycogen storage disease in Japan: clinical manifestations, treatments and long-term outcomes

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Many reports have been published on the long-term outcome and treatment of hepatic glycogen storage diseases (GSDs) overseas; however, none have been published from Japan. We investigated the clinical manifestations, treatment, and prognosis of 127 hepatic GSD patients who were evaluated and treated between January 1999 and December 2009. A characteristic genetic pattern was noted in the Japanese GSD patients: most GSD Ia patients had the g727t mutation, and many GSD Ib patients had the W118R mutation. Forty-one percent (14/34) of GSD Ia patients and 18% (2/11) of GSD Ib patients of ages ⩾13 years 4 months had liver adenoma. Among subjects aged 10 years, 19% (7/36) of the GSD Ia patients and none of the GSD Ib patients had renal dysfunction. The mean height of male GSD Ia patients aged ⩾18 years was 160.8 ± 10.6 cm ($n = 14$), and that of their female counterparts was 147.8 ± 3.80 cm ($n = 9$). Patients with hepatic GSDs develop a variety of symptoms but can survive in the long term by diet therapy, corn starch treatment and supportive care. Liver transplantation for hepatic GSDs is an important treatment strategy and can help improve the patients'quality of life.
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Keywords: adenoma; glycogen storage disease; g727t; height; hepatocellular carcinoma; liver transplantation; renal dysfunction; W118R

INTRODUCTION

Glycogen storage diseases (GSDs) are inherited metabolic diseases caused by the deficiency of enzymes regulating glycogenolysis or gluconeogenesis. As glycogen primarily accumulates in the liver and muscle, the disorders of glycogen degradation affect the liver, muscles or both. Hypoglycemia is the main symptom of hepatic GSDs, whereas muscle weakness or elevated muscle enzyme is the main symptom of myopathic GSDs. Hepatic GSDs, except for GSD IXa, are autosomal recessive, and GSD IXa is an X-linked recessive disorder. GSD Ia, GSD III and GSD IXa account for 80% of hepatic GSDs.

GSD Ia (Mendelian Inheritance in Man (MIM) no. 232200) is caused by a deficiency of glucose-6-phosphatase (EC 3.1.3.9) in the endoplasmic reticulum. GSD Ib (MIM no. 232220) is caused by a deficiency of glucose-6-phosphate transporter, which leads to the dysfunction of glucose-6-phosphatase in the endoplasmic reticulum. GSD Ia is the most common GSD, and its frequency is 1/100 000 to 1/400 000 births in the general Caucasian population; GSD Ib is much less frequent than GSD Ia. The manifestations of GSD Ia are short stature, hypoglycemia, hepatomegaly, hyperlipidemia, hyperuricemia, hyperlactacidemia, hepatoadenoma, renal disorder[1,2] and hepatocellular carcinoma.[3,4] Most GSD Ib patients have neutropenia and neutrophil dysfunction in addition to these symptoms. GSD III (MIM no. 232400) is caused by a deficiency of the debranching enzyme, which consists of amylo-1,6-glucosidase (EC 3.2.1.33) and oligo-1,4-1,4-glucantransferase (EC 2.4.1.25). The incidence of GSD III has been reported to be 1 per 83 000 live births in Europe and 1 per 100 000 live births in North America.[5] There are two major GSD III subtypes: GSD IIIa, which affects both the liver and muscle and accounts for 80% of all GSD III cases, and GSD IIIb, which affects only the liver and comprises approximately 15% of them.[6] The manifestations of GSD III are similar to those of GSD Ia, and many patients with GSD IIIa have hypertrophic cardiomyopathy.[7]

GSD IV (MIM no. 232500) is caused by a deficiency of amylo-1,4 to 1,6-transglucosidase (EC 2.4.1.18), which leads to the absence of branched glycogen. GSD IV, which is the most severe type of GSD, represents 0.3% of all GSDs.[8] This disease rapidly progresses to cirrhosis early in life and causes death between 3 and 5 years of age because of liver failure.[9] If signs of GSD IV, such as cervical cystic hygroma, are detected,[8] the patients are likely to die in the neonatal period. The effective treatment for progressive GSD IV is liver

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transplantation.[10] GSD VI (MIM no. 232700), which is rarer and milder than the other hepatic GSDs, is caused by a deficiency of glycogen phosphorylase (EC 2.4.1.1) in the liver. GSD IXa (MIM no. 306000) is caused by a deficiency of phosphorylase kinase α2 (PHKA2)—a subunit of phosphorylase kinase (EC 2.7.11.19), which consists of four subunits, namely, α, β, γ and δ. The clinical course of GSD IXa is benign, and most adult patients are asymptomatic.[11] With aging, clinical and biochemical abnormalities gradually disappear. The other subtypes of GSD IX include subtypes caused by a deficiency of phosphorylase kinase β, phosphorylase kinase γ or δ, or muscle phosphorylase kinase. The Fanconi–Bickel syndrome, GSD XI (MIM no. 227810), is caused by a deficiency of glucose transport 2 and is characterized by hepatorenal glycogen accumulation and proximal renal tubular dysfunction.[12]

The treatment for these hepatic GSDs comprises the prevention of hypoglycemia. The basic treatment is the consumption of frequent meals and uncooked cornstarch.[13–15] Moreover, restriction of the intake of sugars, such as fructose, galactose, sucrose and lactose, is important mainly for GSD I.

Complete blood glucose control by these measures is unlikely to ameliorate complications, such as hyperuricemia and hyperlipidemia.[16] GSD patients are administered allopurinol for hyperuricemia and statin, fibrates or niacin formulations for hyperlipidemia.[17,18] Administration of angiotensin-converting enzyme inhibitor or/and angiotensin receptor blocker, which have a renoprotective effect, is recommended for GSDs with possible renal complications.[19] Gene therapy can be an effective as a radical treatment measure for GSDs.[20,21] However, the definitive treatment of GSDs is only liver transplantation.[22–25]

Many reports have been published overseas on the long-term outcome and treatment of GSD patients.[11,17,26–28] However, no report has yet been published on the long-term outcome of GSDs in Japan, wherein GSD Ia with a mutation causing mild symptoms has been detected in many cases. We studied the current status of clinical manifestations, treatment, and long-term outcome of hepatic GSDs in Japan.

MATERIALS AND METHODS

Study patients

In 2009, we sent a questionnaire to 928 Japanese institutions, including the departments of pediatrics, endocrinology and metabolism, neonatology, genetics, and transplant surgery, asking doctors if they had diagnosed or provided medical care to hepatic GSDs patients. Each institution was the medical center for a locality and had 300 or more beds. Of the 928 institutions, 668 (72%) responded. Of these 668 institutions, 97 had treated patients with GSDs. A second questionnaire was then sent to these 97 institutions in 2009, and responses were received from 53 (55%) of them. On the basis of the received reports, 127 cases of GSDs diagnosed and treated between January 1999 and December 2009 were studied. We excluded patients who were not definitely diagnosed and considered patients visiting multiple institutions as single patients. The 127 cases of GSDs (types Ia, Ib, III, IV, VI, IXa and others) were diagnosed on the basis of clinical manifestations, family history, enzyme activity, metabolite analysis (75 g oral glucose tolerance test (OGTT) or/and glucagon test) and/or DNA analysis. This study was approved by the ethical committee of the Faculty of Life Science, Kumamoto University.

The definition of clinical manifestations of GSD applied in this study was the same as that proposed by Smit *et al.*[27] In addition, we used the following definitions. Hyperlactacidemia was defined as a blood lactate level $>2.2\,mmol\,l^{-1}$. Hyperuricemia was defined by a history of receiving drugs for hyperuricemia and/or blood uric acid level $>420\,\mu mol\,l^{-1}$. Hyperlipidemia was defined by a history of medical treatment for hyperlipidemia, blood total cholesterol level $>5.9\,mmol\,l^{-1}$, or blood total triglyceride level $>1.7\,mmol\,l^{-1}$. Mental retardation was diagnosed if the patient's intelligence quotient was <70, as per standardized tests, such as the Wechsler Intelligence Scale for Children and the Wechsler Adult Intelligence Scale. Proteinuria was defined by protein levels $>30\,mg\,dl^{-1}$ in 1 spot urea test or $>500\,mg\,day^{-1}$. Renal dysfunction was defined by blood creatinine levels $>90\,\mu mol\,l^{-1}$. Increased susceptibility to infection was defined as a neutrophil count of $<1500/\mu l$ and/or hospitalization more than three times a year because of infection.

Statistical analysis

The age at onset of hepatic GSD patients was expressed in terms of the median and interquartile range, and the age of onset was analyzed by the Mann–Whitney *U*-test of IBM SPSS Statistics Version 19.[29] A *P*-value of <0.05 was considered statistically significant. The height of hepatic GSD patients was expressed in terms of mean ± s.d. values. Kaplan–Meier curves of estimated survival rate were generated by SPSS.

RESULTS

Age at onset and methods for definitive diagnosis of hepatic GSDs

Table 1 indicates the age, onset age and methods used for definitive diagnosis in each of the 127 cases of hepatic GSD. GSD Ib and GSD IV patients manifested symptoms earlier than those with other types of GSD (GSD Ia vs GSD Ib, $P=0.001$; GSD Ia vs GSD IV, $P=0.022$; GSD Ia vs GSD XIa, $P=0.002$). Enzyme activity was measured in 50% (64/127) of the patients with GSDs, and genotype analysis was performed in 50% (63/127); genotypes could be identified in 40% (51/127) of the patients with GSDs. DNA analysis was performed in the case of 52 patients with GSD Ia, 7 patients with GSD Ib, 1 patient with GSD III, 1 patient with GSD VI, 5 patients with GSD IXa and 2 patients with GSD XI. Thereafter, identifiable mutations were detected at a rate of 79% (41/52) in GSD Ia patients, 86% (6/7) in GSD Ib patients, 40% (2/5) in GSD IXa patients and 100% (2/2) in GSD XI patients. Of the GSD Ia patients with recorded identifiable mutations, 81% (29/36) had g727t homozygote mutations and 17% (6/36) had compound heterozygotes with g727t mutations. Of the GSD Ib patients with recorded identifiable mutation, 83% (5/6) had homozygote or compound heterozygote mutations of W118R. Eight patients with GSD Ia, one patient with GSD IXa and one patient with GSD XI were diagnosed by DNA-based and enzymatic analyses.

Clinical manifestations of hepatic GSD

Table 2 indicates the frequency of clinical manifestations in hepatic GSD patients. In GSD Ia patients, growth retardation (78%; 51/65), hypoglycemia (69%; 45/65), hyperuricemia (88%; 57/65) and hyperlipidemia (94%; 61/65) were observed at the frequency of >50% (Table 2a). Convulsions (9%; 6/65), mental retardation (9%; 6/65), liver tumors (22%; 14/65), proteinuria (26%; 17/65), renal dysfunction (11%; 7/65) and increased susceptibility to infection (5%; 3/65) were not frequently observed (Table 2b). Of the 14 GSD Ia patients with liver tumors, 4 had a single adenoma, 9 had 3 or more multifocal adenomas and 1 patient had hepatocellular carcinoma with multiple adenomas. Only one patient with GSD Ia developed acute pancreatitis.

Height of hepatic GSD patients

Figures 1a–d show the height of male and female hepatic GSD patients. The height of 56% (14/25) of the male GSD Ia patients aged <18 years and 43% (6/14) of the male GSD Ia patients aged ⩾18 years was below the third percentile. The mean height of male GSD Ia patients aged ⩾18 years was 160.8 ± 10.6 cm ($n=14$; Figure 1a). Fifty-seven percent (4/7) of the male GSD Ib patients, 50% (2/4) of the GSD III patients aged <18 years and 19% (6/32) of the male GSD IXa patients had heights below the third percentile (Figures 1b and c).

Table 1 Age of onset, diagnosis and definitive diagnosis of hepatic GSD patients

	Patient's age: median (minimum–maximum)	*Age at onset: median (minimum–maximum)*	*Age of diagnosis: median (minimum–maximum)*	*Enzyme activity (%)*	*Identifiable mutation (%)*	*Dead patients*	*Surviving patients*	*No. of patients*
GSD Ia	13 y 8 mo (0 d–11 y 1 mo)	9 mo (0 d–11 y 1 mo)	1 y 2 mo (0 d–11 y 2 mo)	19/65 (29%)	41/65 (63%)	2 (3%)	63 (97%)	65 Patients (male: 41, female: 24)
GSD Ib	12 y 1 mo (1 y–27 y)	3 mo (0 d–4 mo)**	5.5 mo (2 mo–6 y 6 mo)**	3/11 (27%)	6/11 (55%)	1 (9%)	10 (91%)	11 Patients (male: 7, female: 4)
GSD III	12 y (3 y 7 mo–29 y 10 mo)	10.5 mo (7 mo–2 y 3 mo)	1 y (7 mo–2 y 3 mo)	4/6 (67%)	0/6 (0%)	1 (17%)	5 (83%)	6 Patients (male: 4, female: 2)[a]
GSD IV	1 y 1 mo (2 d–14 y 2 mo)	2 mo (0 d–5 mo)*	4 mo (0 d–9 mo)*	4/4 (100%)	0/4 (0%)	3 (75%)	1 (25%)	4 Patients (male: 3, female: 1)
GSD VI	9 y 10 mo (3 y 10 mo–19 y 6 mo)	1 y 3 mo (1 mo–3 y 4 mo)	1 y 4 mo (1 mo–6 y 6 mo)	4/6 (67%)	0/6 (0%)	0 (0%)	6 (100%)	6 Patients (male: 5, female: 1)
GSD IXa	9 y 9 mo (2 y 6 mo–17 y 11 mo)	1 y 7 mo (1 mo–5 y)**	2 y (1 mo–11 y)**	29/32 (91%)	2/32 (6%)	0 (0%)	32 (100%)	32 Patients (male: 32)
Others	11 y 9 mo (11 y 9 mo–29 y 9 mo)	1 y (5 d–1y 6 mo)	1 y 8 mo (1 y 8 mo–1 y 10 mo)	1/3 (33%)	2/3 (67%)	0 (0%)	3 (100%)	3 Patients (male: 2, female: 1)
Total				64/127 (50%)	51/127 (40%)	7 (6%)	120 (94%)	127 Patients (male: 94, female: 33)

Abbreviations: d, days; GSD, glycogen storage disease; mo, months; y, years.
The category 'Others' includes the GSD IX (one patient), other than those with GSD IXa and Fanconi–Bickel syndrome (GSD XI; two patients).
*$P<0.05$.
**$P<0.01$.
[a]Includes four patients each with GSD IIIa (male, 2; female, 2) and two male patients with an unknown subtype.

One hundred percent (5/5) of the male GSD VI patients had height greater than the tenth percentile (Figure 1b). Thirty-three percent (5/15) of the female GSD Ia patients aged <18 years and 44% (4/9) of the female GSD Ia patients aged ⩾18 years had heights below the third percentile. The mean height of female GSD Ia patients aged ⩾18 years was 147.8 ± 3.80 cm ($n=9$; Figure 1d).

Long-term survival of patients with hepatic GSD

Table 1 presents the number of hepatic GSD patients who survived and died. Two patients with GSD Ia (age of death: 6 years 10 months, male; 27 years, female), a male GSD Ib patient (13 years 5 months), a female GSD IIIa patient with cardiomyopathy (24 years 8 months) and a male GSD IV patient (1 year 11 months) died because of liver failure after liver transplantation. The other two patients with GSD IV died of liver failure 2 months after birth.

The long-term survival rate of GSD Ia patients at 20 years after birth was 97% for male patients and 100% for female patients (Figure 2). The survival rate of GSD Ib patients at 20 years after birth was 80% (Supplementary Figure 1).

Treatment for hepatic GSD

Table 3 indicates the treatment received by the hepatic GSD patients. Among the patients with GSD Ia, uncooked corn starch was administered to 98% (64/65) of the patients; allopurinol, to 74% (48/65); lipid-lowering drugs, to 42% (27/65); and angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, to 15% (10/65). Dietary management with restriction of the intake of galactose, fructose and saccharose was used for 63% (41/62) of the patients. Most patients were not taking the GSD formula when they were taking corn starch. Lipid-lowering drugs were administered to 66% (18/27) of the GSD Ia patients with hyperlipidemia and aged 14 years or more. The youngest patient who received lipid-lowering drugs was 5 years old.

Liver transplantation for hepatic GSD

Table 4 shows the ages at which liver transplants were performed for hepatic GSD patients. As many metabolic disorders, such as hypoglycemia, were improved in the two patients with GSD Ia who underwent successful liver transplantation, symptoms such as nasal bleeding and growth disorder were ameliorated. These two patients needed allopurinol, but not diet and corn starch treatment.

Figure 3 and Supplementary Figure 2 present the comparison between the data obtained immediately before liver transplant and 1 year after liver transplant in five patients with GSD Ib. Blood levels of uric acid, total cholesterol and triglyceride in GSD Ib patients improved after liver transplantation, but the abnormalities in the neutrophil count were not ameliorated. All the five patients received granulocyte colony-stimulating factor after liver transplants; however, the frequency of granulocyte colony-stimulating factor administration after liver transplantation was lower than that before transplantation, as was the susceptibility to infection. No patients in this study received bone-marrow transplantation.

DISCUSSION

Most patients with hepatic GSD, except for GSD VI and IXa, which were mild types, manifested symptoms before 2 years of age. Further, the age of onset for GSD Ib and IV was lower than that for the other hepatic GSDs. However, two male GSD Ia patients presented with symptoms at 11 years and 9 years, thereby indicating that GSD may be detected at any age. Enzyme activity in the erythrocytes or leukocytes was measured in patients with GSD III, VI, IXa and XI, without performing invasive liver biopsy. Genome sequencing for GSD III, VI and IXa was difficult and not likely to be performed. Among the GSD I patients, the g727t mutation of the glucose-6-phosphatase gene has been detected in almost 90% alleles of GSD Ia,[30] and the W118R mutation of glucose-6-phosphate transporter gene is highly frequent in GSD Ib patients.[31] Therefore, we performed DNA analysis rather than enzyme assay, which requires invasive liver biopsy in GSD I patients. As this study focused on GSD patients younger than 18 years, we did not include many GSDs patients older than 18 years. Thus, the exclusion of GSD patients older than 18 years and GSD III patients may have introduced a bias in the results.

We investigated the statures of patients with hepatic GSD. Among the hepatic GSDs, GSD I commonly presents with short stature. Height <3 percentile were noted in 56% of the male GSD Ia patients and 33% of female GSD Ia patients aged <18 years. Mean stature in patients with GSD Ia aged >18 years was 160.8 ± 10.6 cm ($n=14$) and 147.8 ± 3.80 cm ($n=9$) for male and female patients, respectively.

Table 2 (a) Frequent manifestations of hepatic GSD; (b) Infrequent manifestations of hepatic GSD

(a)

	Growth disorder	*Hypo- glycemia*	*Hyper- lactacidemia*	*Hyper- uricemia*	*Hyper- lipidemia*	*Hepato- megaly*	*Fatty liver*	*Liver disorder*
GSD Ia	78% (51/65)	69% (45/65)	92% (60/65)	88% (57/65)	94% (61/65)	92% (60/65)	65% (42/65)	97% (63/65)
GSD Ib	55% (6/11)	91% (10/11)	91% (10/11)	64% (7/11)	55% (6/11)	100% (11/11)	64% (7/11)	64% (7/11)
GSD III	50% (3/6)	83% (5/6)	83% (5/6)	67% (4/6)	50% (3/6)	100% (6/6)	50% (3/6)	83% (5/6)
GSD IV	25% (1/4)	50% (2/4)	25% (1/4)	0% (0/4)	0% (0/4)	50% (2/4)	0% (0/4)	50% (2/4)
GSD VI	17% (1/6)	67% (4/6)	50% (3/6)	17% (1/6)	17% (1/6)	100% (6/6)	17% (1/6)	83% (5/6)
GSD IXa	44% (14/32)	34% (11/32)	34% (11/32)	6% (2/32)	41% (13/32)	97% (31/32)	47% (15/32)	84% (27/32)
Others	67% (2/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	67% (2/3)	0% (0/3)	33% (1/3)
Total	61% (78/127)	61% (77/127)	71% (90/127)	56% (71/127)	66% (84/127)	93% (118/127)	54% (68/127)	87% (110/127)

(b)

	Bleeding tendency	*Convulsion*	*Mental retardation*	*Gout*	*Liver tumor*	*Protein- uria*	*Renal dysfunction*	*Hyper- tension*	*Cardio- myopathy*	*Myopathy*	*Osteoprosis*	*Increased susceptibility to infection*
GSD Ia	31% (20/65)	9% (6/65)	9% (6/65)	11% (7/65)	22% (14/65)	26% (17/65)	11% (7/65)	3% (2/65)	6%(4/65)	1.5% (1/65)	3% (2/65)	5% (3/65)
GSD Ib	18% (2/11)	36% (4/11)	27% (3/11)	9% (1/11)	18% (2/11)	9% (1/11)	0% (0/11)	9% (1/11)	9% (1/11)	0% (0/11)	0% (0/11)	100% (11/11)
GSD III	0% (0/6)	67% (4/6)	33% (2/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	17% (1/6)	17% (1/6)	50% (3/6)	0% (0/6)	0% (0/6)
GSD IV	75% (3/4)	0% (0/4)	25% (1/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	50% (2/4)	50% (2/4)	0% (0/4)	25% (1/4)
GSD IV	17% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	17% (1/6)	0% (0/6)	0% (0/6)
GSD IXa	0% (0/32)	0% (0/32)	0% (0/32)	0% (0/32)	0% (0/32)	6% (2/32)	3% (1/32)	0% (0/32)	0% (0/32)	3% (1/32)	0% (0/32)	0% (0/32)
Others	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	33% (1/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)
Total	20% (26/127)	11% (14/127)	9% (12/127)	6% (8/127)	13% (16/127)	16% (20/127)	7% (9/127)	3% (4/127)	6% (8/127)	6% (8/127)	1.6% (2/127)	12% (15/127)

Abbreviation: GSD, glycogen storage disease.

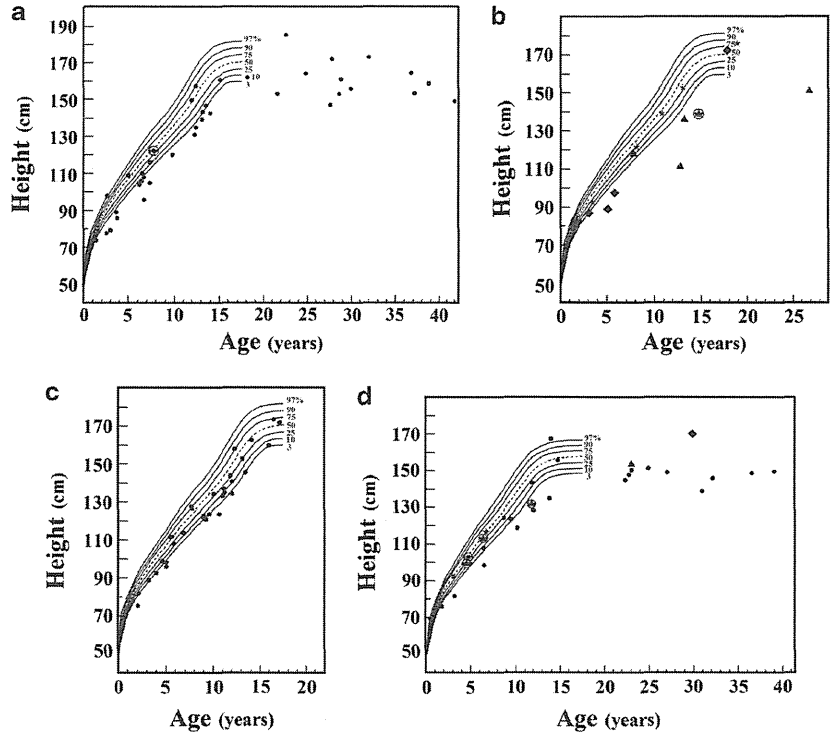

Figure 1 Stature of hepatic glycogen storage disease (GSD) patients. This figure was constructed with the age of GSD patients on the abscissa and the stature of patients with GSD on the ordinate. Percentiles are based on data from Japanese 2000 growth reports provided by the Ministry of Health, Labor and Welfare in Japan. (**a**) Stature of male patients with GSD Ia. The height of the male GSD Ia patient aged 7 years 11 months was measured after liver transplantation. ●: GSD Ia patients ($n=39$); ⊖: patients after liver transplant. (**b**) Stature of male patients with GSD Ib, GSD III and GSD VI. The heights of male GSD Ib patients aged 1 year 10 months and 14 years 10 months were measured after liver transplantation.▲: GSD Ib patients ($n=7$); ◆: GSD III patients ($n=4$); *: GSD VI patients ($n=5$); ⊖: patients after liver transplant. (**c**) Stature of male patients with GSD IXa. ●: GSD IXa patients ($n=32$). (**d**) Stature of female patients with GSD Ia, GSD Ib, GSD III and GSD VI. The heights of female GSD Ia patient aged 4 years 10 months and GSD Ib patients aged 4 years 7 months, 6 years 6 months and 11 years 11 months were measured after liver transplantation.●: GSD Ia patients ($n=24$), ▲: GSD Ib patients ($n=4$), ◆: GSD III patients ($n=1$), *: GSD VI ($n=1$), ⊖: patients after liver transplant.

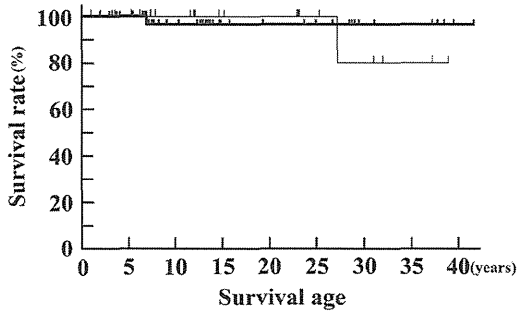

Figure 2 Long-term survival rates in patients with glycogen storage disease (GSD) Ia. The survival rates of 63 patients with different ages are shown by Kaplan–Meier survival curves. Two GSD Ia patients aged 6 years 10 months (male) and 27 years (female) died of liver failure after liver transplant. Male GSD Ia patients (black bold line), $n=41$; female GSD Ia patients (black fine line), $n=22$.

Therefore, we can expect that the final stature of patients with GSD Ia ranges from 3 to 10 percentile of the Japanese height.

Liver tumor and renal dysfunction, which are not frequently observed, are important determinants of the prognosis in patients with GSD.[1] It has been reported that liver adenomas are detected in 22 to 75% of patients with GSD Ia,[28,32] and some of these adenomas developed to hepatocellular carcinoma.[33,34] In this study, liver tumors, which have been reported to be less frequent overseas, were detected in 22% (14/65) of the patients with GSD Ia and in 18% (2/11) of patients with GSD Ib. The youngest GSD Ia patient with liver adenoma was a male patient aged 13 years 4 months, and 41% (14/34) of GSD Ia patients older than this patient had liver adenoma. Nakamura *et al.*[35] reported that 57.9% (11/19) of adult GSD Ia patients with the g727t homozygote mutation had liver adenomas, and 16% (3/19) of them had hepatocellular carcinoma. In this study, only one patient developed hepatocellular carcinoma, which was treated by percutaneous ethanol injection therapy and radiofrequency ablation, and did not recur.

Proteinuria, which is detected in many patients with GSD I, may progress to renal dysfunction or renal failure. In this study, two of the seven GSD Ia patients with renal dysfunction underwent hemodiafiltration. Chen *et al.* reported that 70% of GSD Ia patients aged >10 years presented with renal dysfunction and that 40% of GSD Ia patients with renal dysfunction developed progressive renal failure. The incidence of renal dysfunction, which was 11% (7/65) in GSD Ia patients of this study and 19% (7/36), in GSD Ia patients >10 years old, was very low.

As GSD Ia with g727t mutation is considered to be a mild type of GSD Ia, patients with the g727t mutation may develop only proteinuria but are not likely to develop renal dysfunction. It has been reported that transforming growth factor-β expression increases in the tubular epithelial cells and is involved in the pathophysiology of

Table 3 Treatment for hepatic GSD

Treatment	Dietary management	Uncooked corn starch	Sodium and potassium citrate	Allopurinol	Lipid- lowering drugs	ARB or ACE-I	Hypoglycemic medication	L- carnitine	G-CSF
GSD Ia	63% (41/65)	98% (64/65)	37% (24/65)	74% (48/65)	42% (27/65)	15% (10/65)	6% (4/65)	0% (0/65)	0% (0/65)
GSD Ib	64% (7/11)	82% (9/11)	0% (0/11)	9% (1/11)	9% (1/11)	0% (0/11)	9% (1/11)	27% (3/11)	55% (6/11)
GSD III	33% (2/6)	67% (4/6)	17% (1/6)	17% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
GSD IV	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)
GSD VI	17% (1/6)	67% (4/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/6)
GSD IXa	16% (5/32)	50% (16/32)	0% (0/32)	0% (0/32)	31% (10/32)	0% (0/32)	0% (0/32)	0% (0/32)	0% (0/32)
Others	33% (1/3)	67% (2/3)	33% (1/3)	0% (0/3)	33% (1/3)	0% (0/3)	0% (0/3)	67% (2/3)	0% (0/3)

Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; GSD, glycogen storage disease; G-CSF, granulocyte colony-stimulating factor.

Table 4 Age at liver transplant for hepatic GSD

Age	*<1 y*	*1 y to <6 y*	*6 y to <12 y*	*12 y to <18 y*	*⩾18 y*	*Total*
GSD Ia	0	3	0	0	1	4
GSD Ib	0	4	1	2	0	7
GSD III	0	0	0	0	1	1
GSD IV	2	0	0	0	0	2
Total	2	7	1	2	2	14

Abbreviations: GSD, glycogen storage disease; y, years.

renal interstitial fibrosis, which results from the increase in the expression of extracellular matrix proteins in GSD I patients.[36] Angiotensin receptor blocker, angiotensin-converting enzyme inhibitor and alloprinol have been considered drugs with the highest potential of interfering with transforming growth factor-β expression because the renin angiotensin–aldosterone system and uric acid have been known to involved in the expression of transforming growth factor-β.[37,38] Moreover, it has been recognized that the small, dense low-density lipoprotein and modified low-density lipoprotein induce the development of glomerular sclerosis and renal dysfunction.[39]

Liver tumor is related to constant stimulation by hormones, such as insulin and glucagon, by persistent peripheral hypoglycemia. Therefore, the expression of renal dysfunction and liver tumor negatively correlates with metabolic control.[40] Important treatment strategies are restriction of the intake of galactose, fructose, and saccharose and blood glucose control by consumption of frequent meals and uncooked cornstarch.[40] Moreover, allopurinol, lipid-lowering drugs, and angiotensin receptor blocker or angiotensin-converting enzyme inhibitor have been reported to be significantly important in delaying the progression of kidney disease in GSD I patients.[19,39,41]

Recent reports have indicated that GSD patients may present with diabetes. Two GSD Ia patients who were brothers and had the g727t homozygote mutation developed type II diabetes and received therapy involving an α-glucosidase inhibitor and an insulin secretagogue. They monitored themselves for hypoglycemia attacks and corrected the same by consuming food or glucose. As shown in Table 1 and Figure 2, patients with hepatic GSD, except for those with GSD IV, can survive in the long term. Further, reports have also shown that GSD Ib and GSD III patients developed type II diabetes.[42,43] Therefore, physicians must pay attention to the development of obesity- and lifestyle-related diseases in GSD patients.

Table 3 indicates the treatments received by patients with hepatic GSD. As treatment after liver transplantation was recorded in Table 3,

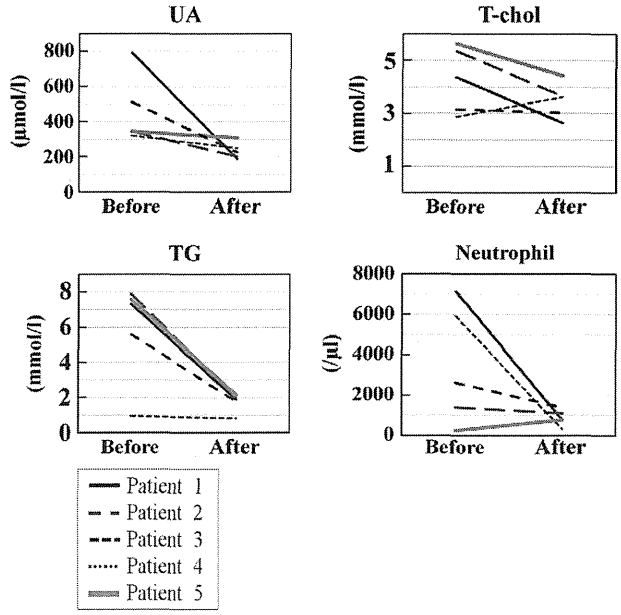

Figure 3 Comparison of data immediately before and 1 year after liver transplantation in glycogen storage disease (GSD) Ib patients. The age at liver transplantation was 1 year 1 month in patient 1 (male), 3 years 6 months in patient 2 (female), 3 years 6 months in patient 3 (male), 3 years 11 months in patient 4 (female) and 8 years 6 months in patient 5 (female). Only patient 3 received allopurinol after liver transplant. T-chol, total cholesterol; TG, triglyceride; UA, uric acid.

none of patients with GSD IV take dietary treatment and corn starch treatment. Use of lipid-lowering drugs has been recommended for adult GSD patients overseas.[18] Although definitive criteria for the use of lipid-lowering drugs in Japan have not yet been established, the youngest patient who received hypoglycemic medication was 5 years old.

Fourteen patients with hepatic GSD received liver transplants. According to overseas reports, the indications for liver transplantation in GSD patients are the progression of adenomatous lesions or multiple adenomas, suspicion or detection of malignant transformation of an adenoma, unresponsiveness to medical therapy, insufficient control of hypoglycemia, and growth or sexual retardation.[17,24,44] In Japan, the definitive criteria for liver transplants are controversial; many pediatricians and transplant surgeons follow the same indications reported overseas for liver transplantation. GSD I patients with uncontrolled hypoglycemia, which leads to convulsions and mental retardation, should receive liver transplants. Ninety-one

percent (10/11) of patients with GSD I received liver transplants because of insufficient control of hypoglycemia and metabolic disorders, despite medical therapy. GSD III and GSD IV patients received liver transplants because of liver failure, which was considered an indication of liver transplant, as per the pediatric end-stage liver disease scores. In this study, all GSD I patients with multiple liver adenomas underwent hepatectomy, and only one patient with GSD I received a liver transplant because of adenoma recurrence after adenoma resection. Five of 14 GSD patients died because of liver failure <2 months after liver transplantation. The other nine patients survived and improved such that they did not develop hypoglycemia without medication and showed better increase in height. The frequency of infection decreased in GSD Ib patients after transplantation, as described previously.[45] Liver transplants contributed to an improved quality of life (QOL) in GSD patients. We believe that liver transplants should be proactively performed in patients with GSD Ib. Although the success rate of liver transplantation for hepatic GSD in this study was lower than that reported abroad,[24,46–49] the low success rate of liver transplants may be attributed to the severe liver failure in the fatal GSD cases before transplantation.

In conclusion, we discussed the diagnosis, treatment and long-term outcome of hepatic GSDs and the present status of hepatic GSD patients in Japan. We found a characteristic genetic pattern with many GSD Ia patients presenting with the g727t mutation and GSD Ib patients showing the W118R mutation. Although patients with hepatic GSD, except for those with GSD IV, develop a variety of symptoms, they can survive in the long-term by diet therapy, corn starch treatment and supportive care. Liver transplantation is an important therapeutic strategy for hepatic GSD and can help improve the patients' QOL.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

of 12 autopsy subjects with infantile polyarteritis nodosa,[13,15] now considered indistinguishable from KD. Although scrotal redness and tenderness are important signs of testicular torsion, careful observation should be made to avoid unnecessary surgical exploration. Color Doppler imaging and radionuclide testicular scanning may be helpful to differentiate epididymo-orchitis or hydrocele from testicular torsion. A similar suspected pathogenesis was discussed in a report on acute scrotum in Henoch–Shönlein purpura; acute scrotum is a relatively well-known complication of this disease.[12]

Five of the 10 reviewed patients and the two present patients had edema of the extremities. Although data on serum albumin level were available only for the present two patients, both patients had low serum albumin. This suggested an association between increased vascular permeability in acute phase of the disease and acute scrotum. The present two patients had acute scrotum after diagnosis of KD. In contrast, eight of 10 reviewed patients had acute scrotum on admission or before diagnosis, suggesting the diagnostic value of this condition.

Although the incidence of acute scrotum in patients with KD is unknown, careful observation may identify additional patients with the complication. Most of the reported patients were free of tenderness and the condition resolved spontaneously over time, suggesting the potential presence of overlooked patients with this complication of KD.

In summary, based on the 10 reported cases and the two present cases, acute scrotal symptoms in KD may be extracardiac findings of the acute phase of the disease, and must be reported in the list of possible KD complications.

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VLCAD deficiency in a patient who recovered from ventricular fibrillation, but died suddenly of a respiratory syncytial virus infection

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Abstract VLCAD deficiency is an autosomal recessive disorder caused by a defect of fatty acid oxidation. The phenotype is classified into three clinical forms on the basis of the onset of symptoms: a severe form with neonatal onset; a milder form with childhood onset; and a late-onset form. The neonatal form is the most common, and has a higher mortality rate

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than the others. We report the case of a newborn infant with VLCAD deficiency who developed ventricular fibrillation, which was successfully treated by intensive care, but who suddenly died after a respiratory syncytial virus infection. Early institution of i.v. glucose treatment and active immunization with vaccine, such as palivizumab (anti-RSV mAb), may be important to reduce the frequency and severity of life-threatening episodes.

Key words arrythmia, fatty acid oxidation disorder, neonatal sudden death, respiratory syncytial infection, VLCAD deficiency.

Patients with fatty acid oxidation disorders may present with early onset of a severe form usually associated with cardiomyopathy and leading to sudden death in some cases. In infants, the disease course can be rapid and is difficult to diagnose in the emergency department. Very-long-chain acyl-coenzyme A (CoA) dehydrogenase (VLCAD) deficiency is an autosomal recessive disorder caused by a defect of ACADVL gene affecting fatty acid oxidation. The prevalence of the disease has been estimated to be 1 in 150 000. Symptoms of VLCAD deficiency appeared during infancy or childhood are hypoglycemia, lethargy, muscle weakness, liver failure and heart failure. Here, we report the case of a newborn infant with VLCAD deficiency who developed ventricular fibrillation, which was successfully treated by intensive care, but who suddenly died after a respiratory syncytial virus infection.

Case report

The present patient was a boy weighing 3566 g at birth who was born at 39 weeks 4 days of gestation following an unremarkable pregnancy. There was no significant family history or consanguinity. On the first day of life, tachypnea and grunting were noted. These findings suggested pneumonia, but the patient's clinical condition did not improve with i.v. antibiotics. The patient did not respond well and was therefore transferred to the pediatric emergency center for further examination. He had slightly delayed capillary refilling time, oxygen saturation of 99%, heart rate 118 beats/min, and respiratory rate 80 breaths/min. Laboratory analysis indicated blood glucose and potassium levels of 42 mg/dL (2.33 mmol/L) and 7.05 mmol/L, respectively, and blood gas measurement showed metabolic acidosis with pH 7.294, pCO_2 29.4 mmHg, pO_2 35.6 mmHg, HCO_3 13.8 mmol/L, base excess −11.1 mmol/L, and anion gap 25.2 mEq/L. Electrocardiograph (ECG) monitoring indicated a sudden onset of ventricular fibrillation (VF) (Fig. 1a). Cardiac pulmonary resuscitation was attempted, with calcium gluconate and epinephrine, and after 30 min the patient showed recovery to sinus rhythm. Sodium bicarbonate followed by glucose-insulin therapy was initiated. The patient was then transferred to the neonatal intensive care unit (NICU) at Kumamoto University Hospital. After arrival, hypoglycemia, hyperkalemia, and metabolic acidosis recovered quickly. Cardiac function required more time for complete recovery, but the patient did not experience arrhythmia. Acylcarnitine analysis on tandem mass spectrometry (MS/MS), using a dried blood spot taken on admission, indicated elevated long-chain acylcarnitines, with a C14-1-acylcarnitine level of 4.08 μmol/L (control, <0.40; Table 1).

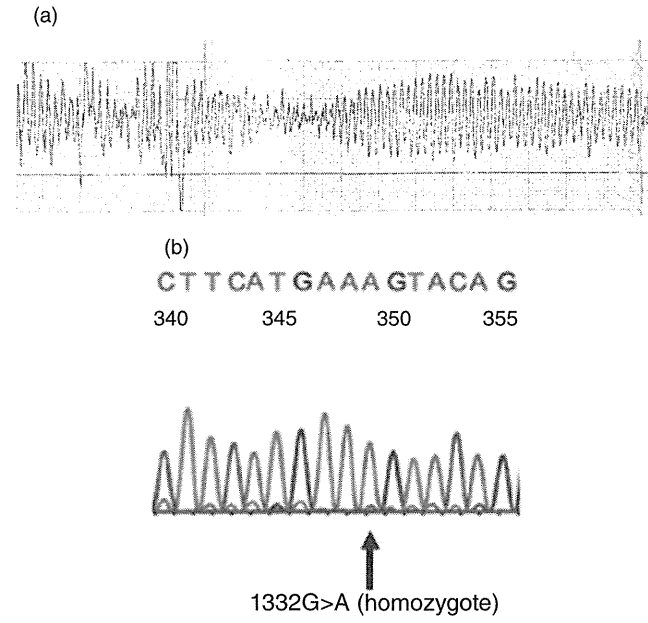

Fig. 1 (a) Electrocardiograph monitoring at the onset of ventricular fibrillation. (b) Gene analysis of the acyl-CoA dehydrogenase, very-long-chain (ACADVL) gene, indicating a homozygote c.1332G>A mutation in the exon–intron junction.

Table 1 Acylcarnitine concentration on MS/MS

Acylcarnitine	Concentration (nmol/mL)	Control (nmol/mL)
C0	27.86	>10
C2	9.59	21.16 ± 5.26 (mean ± SD)
C4	0.15	<1.0
C5	0.13	<1.0
C6	0.06	<0.30
C8	0.17	<0.30
C10	0.78	<0.35
C12	1.09	<0.35
14	6.15	<0.40
C14:1	4.08	<0.40
C16	13.38	<6.0
C16-OH	0.12	<0.05
C18	2.64	<3.0
C18:1	3.3	<3.0
C18:1-OH	0.05	<0.05

MS/MS, tandem mass spectrometry.

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Table 2 A VLCAD activity assay†

Subject	Palmitoyl-CoA dehydrogenase activity (pmol/min/10^6 lymphocytes)
Patient	0.42
Control	25.1
Normal (n = 31)	54.5 ± 17.5

†Using lymphocytes and palmitoyl-CoA as a substrate. VLCAD, very-long-chain acyl-coenzyme A (CoA) dehydrogenase.

These data suggested that the newborn patient may have very-long-chain acyl-coenzyme A (CoA) dehydrogenase (VLCAD) deficiency; enzyme assay[1] was then performed. Palmitoyl-qCoA dehydrogenase activity in lymphocytes was reduced to approximately 1% that of the mean in normal control subjects (Table 2). The diagnosis of VLCAD deficiency was confirmed on these findings. Gene analysis identified a homozygote c.1332G>A mutation in the exon–intron junction of the acyl-CoA dehydrogenase, very-long-chain (ACADVL) gene (Fig. 1b), indicating a splicing abnormality. After confirming the diagnosis, the patient had normal development with long-term dietary therapy and supplementation of L-carnitine and medium-chain triglyceride (MCT) oil. After the neonatal period, echocardiography and ECG were normal. Vomiting and diarrhea were sometimes associated with metabolic acidosis, but the patient recovered quickly after rapid transfusion of glucose and electrolytes. The day before his death at 2 years old, he had cough and low-grade fever. He presented to the emergency department, which he often visited for regular treatment. He was diagnosed as having respiratory syncytial virus (RSV) infection according to the RSV detection kit. Given that his respiratory condition was satisfactory and blood gas analysis was normal, it was decided that hospitalization was not necessary. He was therefore returned his home with some medicine for cough and fever, but he had only half the usual quantity of MCT milk that night. He coughed and woke up early in the morning, and he was conscious until just before the attack. His breathing sounded normal, then he suddenly stood up and fell down and became unconscious. He seemed to be in cardiopulmonary arrest when he arrived at the emergency department. After efforts at resuscitation we confirmed his death at the hospital. A cardiogenic cause, particularly arrhythmia, was most likely the cause of the sudden death. The death was too sudden to be due to breathing problems or brain lesion. Because there was no sign of vomiting, no sign of abuse, no congestion due to suffocation, we speculated that his death was due to arrhythmia induced by VLCAD deficiency.

Discussion

VLCAD deficiency is an autosomal recessive disorder and the prevalence of VLCAD deficiency has been estimated to be 1 in 150 000. The phenotype of VLCAD deficiency is heterogeneous. It is classified into three clinical forms on the basis of the onset of symptoms: a severe form with neonatal onset; a milder form with childhood onset; and a late-onset form. The neonatal form is the most common, and patients present with cardiomyopathy, hepatopathy, and skeletal myopathy. This form has a higher mortality rate than the others.[2] VF and respiratory arrest have been reported in patients who develop VLCAD deficiency within 1 year of birth.[3] In the present case, the patient developed VF and was rescued by cardiopulmonary resuscitation, because the pediatrician was at his bedside during the development of VF. When the patient was transferred to NICU, metabolic acidosis was improved by glucose transfusion. First, we suspected mitochondrial disease and secondary cardiac disorder. MS/MS was very useful for the final diagnosis of VLCAD deficiency.

In Kumamoto, MS/MS analysis was initiated as a pilot study 5 years ago, and MS/MS was introduced for mass screening of newborns with approximately 100% agreement. Because clinical manifestations in the present case were observed 2 days after birth, the patient was not covered by standard screening. The abnormality was detected only when post-symptom high-risk screening was performed. Elevations in C14:1, C16, and C16+18/C2 were identified on MS/MS, and VLCAD deficiency was suspected. At this point, the patient was given MCT milk and carnitine. Next, we performed a fatty acid β-oxidation assay and found that the metabolism of C14 to C12 was abnormal. We also performed a VLCAD enzyme assay and ACADVL gene analysis.[1] Palmitoyl-CoA dehydrogenase activity in the present patient was found to be severely decreased. Molecular analysis of the ACADVL gene encoding VLCAD showed that the patient had a single base mutation, c,1332G>A, at the exon–intron junction. To the best of our knowledge, this case presents a novel mutation. We examined the sequences by calculating splicing score (http://www.fruitfly.org/seq_tools/splice.html). In the normal sequence (CTTCATGAAGGTACAGGACGGT), splice site was recognized with donor score 0.9. The false-positive (FP) rate and the correlation coefficient (CC) were 1.1% and 0.73. In the present patient's sequence (CTTCATGAAAGTACAGGACGGT), the splicing was not recognized. As a result of the mutation, abnormal splicing of the mRNA would occur. We assumed that it was a mutation causing exon-skipping or connection to a new junction.[4]

An inborn error in metabolism is one of the differential diagnoses of unknown cardiomyopathy or arrhythmia. In this case, MS/MS was insufficient for preclinical diagnosis because of the delayed time of sampling to detect early-onset VLCAD, but it was very useful for accurate diagnosis.[5] It is possible to prevent secondary complications of VLCAD with intake of MCT milk and carnitine supplementation and with diet therapy. In past reports, patients surviving the initial episode have nearly normal cardiac function by avoidance of fasting, and using a low-fat diet with frequent meals and vigilance during intercurrent illness.[6] We can expect normal development with careful follow up for most patients.[7] It is important to start a glucose infusion, not only in cases of gastroenteritis and starvation, but also in cases of general infection with the potential for exacerbation.

The prognosis for control of VLCAD deficiency is very challenging, even after successful resolution of several crises. Early institution of i.v. glucose treatment may be important to

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reduce the frequency and severity of life-threatening episodes. In addition, active immunization with vaccine, such as palivizumab (anti-RSV mAb), might be necessary.

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Transverse myelitis and acute motor sensory axonal neuropathy due to *Legionella pneumophila*: A case report

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Abstract Guillain–Barré syndrome is a rapidly progressive symmetrical muscle weakness associated with acute inflammatory disease. Transverse myelitis (TM) is the inflammation of the spinal cord characterized by rapidly evolving muscle weakness in the lower extremities, defects in sensory level and sphincter dysfunction. Guillain–Barré syndrome, and TM association occurs very rarely in childhood. A 7-year-old girl presented with complaints of neck pain, spout-style vomiting, cough, shortness of breath, and acute paraparesis with sensory and sphincter disturbance. The patient was intubated because of increased respiratory distress. A positive direct fluorescein antigen test in bronchoalveolar lavage confirmed *Legionella pneumophila* infection. Imaging and neurophysiologic studies were diagnostic for TM with acute motor and sensory axonal neuropathy. She was treated with a combination of high-dose methylprednisolone and intravenous immunoglobulins, and we observed incomplete recovery. The presented case is the first child with concomitant TM and acute motor and sensory axonal neuropathy related to *L. pneumophila* infection.

Key words acute motor and sensory axonal neuropathy, child, immune modulation, *Legionella pneumophila*, transverse myelitis.

Demyelinating disorders can affect any part of the nervous system. Transverse myelitis (TM), which is characterized by focal spinal cord inflammation, may be idiopathic, parainfectious or disease-associated. Diseases associated with TM include demyelinating conditions and connective tissue disorders. Apart from TM attributable to direct spinal cord infection, TM is autoimmune. Demyelination limited to the spine is known as TM, whereas radicular and peripheral nerve demyelination is recognized as the acute inflammatory demyelinating form of Guillain–Barré Syndrome (GBS).[1,2] Acute motor and sensory axonal neuropathy (AMSAN), a subtype of GBS, is an autoimmune and usually post-infectious disease characterized by endoneurial inflammation with both primary demyelination and axonal degeneration.[2]

To our knowledge, this is the first presentation of a child with simultaneous TM and AMSAN related to *Legionella pneumophila* infection in the English-language medical literature.

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p.E66Q mutation in the *GLA* gene is associated with a high risk of cerebral small-vessel occlusion in elderly Japanese males

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See editorial by Meschia on page 3.

Keywords:
cerebral hemorrhage, cerebral infarction, cerebral small-vessel occlusion, Fabry disease, *GLA*, lacunar infarction, risk factors, α-galactosidase A

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Background and purpose: *GLA* is the causative gene of Fabry disease, an X-linked lysosomal storage disorder resulting from α-galactosidase A (α-GAL) deficiency. Stroke is an important manifestation of Fabry disease, and recent epidemiological studies have indicated that up to 4.9% of young male cryptogenic stroke patients have *GLA* mutations. To determine the importance of *GLA* mutations in the general stroke population, the frequency of *GLA* mutations in Japanese male ischaemic stroke (IS) patients with various risk factors and ages was measured.

Methods: A total of 475 male IS patients (mean age 69.7 ± 12.5 years), were enrolled in this study. A blood sample was obtained to produce blood spots for measurement of α-GAL activity. Blood samples with decreased enzymatic activity were reassayed and the entire *GLA* gene was analyzed by direct DNA sequencing if α-Gal A activity was consistently low.

Results: α-Gal A activity was decreased in 10 men, five of whom (1.1%) had the *GLA* gene mutation, p.E66Q. All IS patients with p.E66Q mutation had substantial residual α-Gal A activity, in contrast to patients with classic-type Fabry disease. Clinically, all patients with p.E66Q mutation were > 50 years old and had multiple small-vessel occlusions (lacunar infarctions). Statistical analysis using Fisher's exact test showed the allele frequency of *GLA* p.E66Q in patients with small-vessel occlusion to be significantly higher than that in the general Japanese population [odds ratio (OR) = 3.34, $P = 0.025$).

Conclusions: *GLA* p.E66Q mutation is a genetic risk factor for cerebral small-vessel occlusion in elderly Japanese males.

Introduction

Fabry disease (MIM301500) is an X-linked lysosomal storage disorder resulting from deficiency of α-galactosidase A (α-Gal A) [1]. The enzymatic defect leads to progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids in the vascular endothelial lysosomes of the kidneys, heart, brain, and skin. Affected males who have little or no detectable α-Gal A activity exhibit the classic phenotype with onset of angiokeratoma, acroparesthesia, and hypohidrosis in childhood. With advancing age, the occurrence of renal failure, cardiac disease, and stroke lead to a decline in activities of daily living and premature death. Stroke is one of the major complications of classic Fabry disease [2] and has been described in 6.9–24.2% of patients [3–5]. Estimates of the prevalence of classic Fabry disease vary from 1 in 40 000 to 1 in 60 000 [1,6].

On the other hand, patients with substantial levels of residual α-Gal A activity have late-onset milder phenotypes, including renal [7] and cardiac [8] variants. Recent studies involving newborn screening for α-Gal A activity in Fabry disease showed surprisingly high incidences of mutations of 1 in 1250–3100 male infants [9,10]. Most mutations found in newborn screening were associated with the late-onset variant phenotype, suggesting that many patients with these

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GLA mutations are under-diagnosed. Screening for Fabry disease in high-risk populations identified previously undiagnosed Fabry patients in 0.2–1.2% of males undergoing hemodialysis [7,11,12] and 0.9–4% of males with left ventricular hypertrophy or hypertrophic cardiomyopathy [8,13,14]. Furthermore, the prevalence of unrecognized Fabry disease in young male patients with stroke was reported to be up to 4.9% [15–20]. However, most stroke patients are elderly and there has been only one population-based study in unselected patients with stroke [21]. It is likely that *GLA* mutation is itself a risk factor for accelerated atherosclerosis and cardiac and renal disease, which can lead to emboli and hypertension, and therefore unrecognized Fabry patients may be found amongst elderly stroke patients [22]. To determine the importance of *GLA* mutations in the general stroke population, the frequency of *GLA* mutations in Japanese male ischaemic stroke (IS) patients with various risk factors and ages was measured.

Methods

Patients

Fifteen clinical neurology departments in Nagano prefecture, Japan, participated in this prospective cross-sectional study. From August 2007 to December 2011, 475 male patients aged 20–91 years (mean ± SD, 69.7 ± 12.5 years), presenting consecutively at a participating neurology department with IS, were enrolled in this study. Patients who were unable to provide informed consent or who had already been diagnosed with Fabry disease were excluded from the study. This study was approved by the Ethical Committee of Shinshu University School of Medicine and the ethics committees of each of the participating clinical neurology centers, and written informed consent was obtained from each patient prior to enrollment. After informed consent was obtained, demographic data, cerebrovascular risk factors, presence of signs and symptoms of Fabry disease, and clinical and neuroimaging data were registered in a database using case report forms. Assessment of clinical symptoms and signs suggestive of Fabry disease was performed in all patients with *GLA* gene mutations. Screening for angiokeratoma was performed by routine clinical examination, and the presence of acroparesthesia and hypohidrosis was determined by anamnesis. In addition, cardiac function tests, including serum brain natriuretic peptide (BNP) and human atrial natriuretic peptide (hANP) concentrations, chest roentgenography, electrocardiography, and echocardiography, and renal function tests, including routine urine test and determination of serum creatinine and blood urea nitrogen (BUN) levels, were performed in all patients with *GLA* mutation.

α-Gal A enzyme assay and mutation analysis

A blood sample was obtained for production of blood spots for measurement of α-Gal A activity. α-Gal A activity was determined using a fluorescent substrate as described previously [23]. Briefly, 40 μl of McIlvan buffer (0.1 M citrate, 0.2 M NaH_2PO_4, 36.8:63.2, pH 6.0) was added to each well of 96-microwell plates. Three-millimeter punch specimens of dried blood spots were added to the buffer and processed for extraction at room temperature for 2 h. Aliquots of 30 μl of blood extract were transferred to fresh 96-microwell plate. An aliquot of 100 μl of the reaction mixture (3.5 mM 4-MU galactosylpyranoside, 100 mM citrate, 200 mM phosphate, 100 mM *N*-acetylgalactosamine) was added to each well of the microwell plates and incubated at 37°C for 24 h. The reaction was terminated with 150 μl of termination solution (300 mM glycine, NaOH, pH 10.6) immediately after the reaction. Fluorescence intensity from the 4-methylumbelliferones in the wells was measured with a fluorescence plate reader (BIO-TEK, Winooski, VT, USA) at 450 nm. One unit (1 AgalU) of enzymatic activity was equal to 0.34 pmol of 4-methylumbelliferyl-D-galactopyranoside cleaved/h per disc. Blood samples with decreased enzymatic activity (< 17 AgalU) were reassayed.

If blood αGal A activity was consistently low, the entire *GLA* gene was analyzed. For DNA analysis, total genomic DNA was extracted from leukocytes of patients. All seven exons and the flanking intronic sequences of the *GLA* gene were amplified by PCR, and the amplification products were analyzed by direct sequencing (Fig. 1).

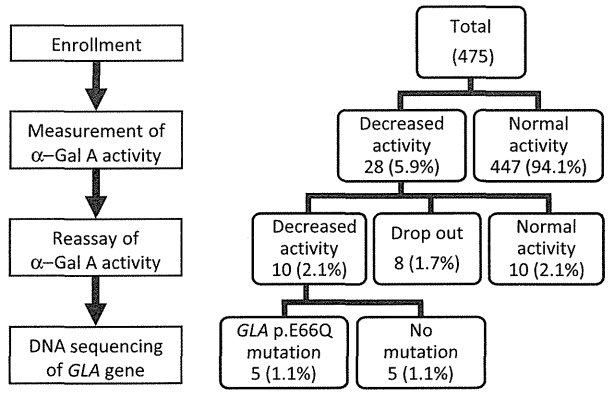

Figure 1 Flowchart for the present study.

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Results

Clinical data of enrolled patients

Table 1 summarizes the demographic characteristics of the enrolled patients. The mean age (±SD) in the cohort of 475 patients participating in this study was 69.7 ± 12.5 years. Stroke etiology was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [24] as large-artery atherosclerosis in 114 patients (24.0%), cardioembolism in 79 (16.6%), and small-vessel occlusion (lacunar infarction) in 240 (50.5%). Stroke of other determined etiology was present in 12 patients (2.5%): cervicocephalic arterial dissection ($n = 4$) and paraneoplastic coagulopathy ($n = 8$). Stroke of undetermined etiology was present in 61 patients (12.8%). Subtypes of IS overlapped in 31 patients, as they had histories of multiple ISs with different subtypes.

Diagnostic test results

The average α-Gal A activity of the study population was 27.7 ± 10.7 AgalU. The distribution of α-Gal A activity in the whole study population is shown in Fig. 2. Initial screening for α-Gal A activity in blood spots from 475 male patients with stroke detected 28 (5.9%) patients with enzyme level below the normal cut-off value of 17.0 AgalU. A repeat blood spot was obtained from 20 patients. When retested, 10 (2.1%) patients had α-Gal A activities < 17.0 AgalU, whereas the other 10 (2.1%) had normal enzyme activities (≥ 17 AgalU). DNA sequencing of *GLA* was performed in these 10 doubly screened-positive patients with low α-Gal A activity, and *GLA* gene mutation was identified in five (1.1%) patients. All five patients had the same missense mutation, a single base sequence change (c. 196G>C), causing substitution of a glutamate reside with glutamine at codon 66 (p.E66Q). The average α-Gal A activity of the stroke patients with the p.E66Q mutation was 11.3 ± 1.6 AgalU (range 9.8–13.5 AgalU), which was relatively high compared with patients with classic-type Fabry disease.

Clinical data of patients with *GLA* p.E66Q mutation

The clinical, biochemical, and molecular features of the patients with the *GLA* p.E66Q mutation are summarized in Table 2. All patients with the p.E66Q mutation were older than 50 years, with a mean age of 69.6 ± 12.5 years. All patients had multiple small-vessel occlusions (Fig. 3), which were accompanied by white matter lesions (leukoaraiosis) in three patients

Table 1 Demographic and characteristics of the patient population

		Subtypes of IS[a]				
	All IS	Large-artery atherosclerosis	Cardioembolism	Small-vessel occlusion	Stroke of other determined etiology	Stroke of undetermined etiology
Number of patients	475	114	79	240	12	61
Age (mean ± SD)	69.7 ± 12.5	70.4 ± 11.0	74.1 ± 12.9	68.5 ± 12.1	67.3 ± 17.2	73.6 ± 13.2

[a]Subtypes of IS overlapped in 31 patients, as they had episodes of multiple IS with different subtypes.

Figure 2 The distribution of α-galactosidase A (α-Gal A) activity in all the patients included in this study. The *x*-axis indicates age (years) and the *y*-axis indicates α-Gal A activity (AgalIU). The cut-off α-Gal A activity was 17 AgalIU. Filled diamonds, open triangles, open squares, and open circles indicate individuals with normal enzymatic activity in dried blood spot screening, low enzymatic activity without *GLA* gene mutations, low enzymatic activity without DNA analysis, and low enzymatic activity with *GLA* p.E66Q mutation, respectively.

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Table 2 Clinical features of patients with *GLA* gene mutation

Patient	Age	Sex	α-Gal A activity (AGalU; normal > 17)	*GLA* gene mutation	Type of cerebral infarction		Cerebral hemorrhage	Risk factor for stroke	Other complications of Fabry disease
1	70	M	11.6	p.E66Q (c.196G>C)	Multiple	Lacunar	Thalamic hemorrhage	–	–
2	83	M	12.0	p.E66Q (c.196G>C)	Multiple	Lacunar/ atherothrombotic/ leukoaraiosis	Putaminal hemorrhage	Hypertension (good control)	–
3	76	M	13.5	p.E66Q (c.196G>C)	Multiple	Lacunar/leukoaraiosis	Multiple microbleeds	–	–
4	66	M	9.8	p.E66Q (c.196G>C)	Multiple	Lacunar/leukoaraiosis	Symptomatic hemorrhage[a]	–	–
5	50	M	9.8	p.E66Q (c.196G>C)	Multiple	Lacunar	–	Hypertension (good control)	–

α-Gal A, α-galactosidase A;
[a]Location of the hemorrhage was unknown.

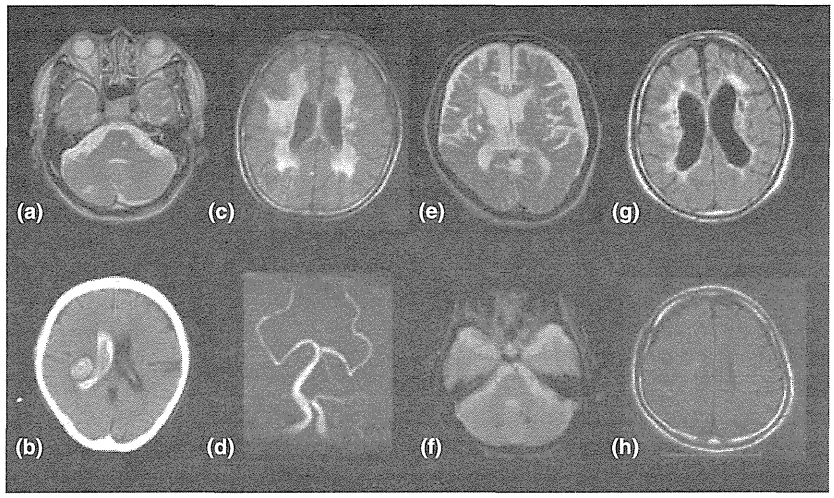

Figure 3 Brain MRI and CT findings of patients with *GLA* p.E66Q. (a, b) Patient 1. T2-weighted MRI showed small-vessel occlusions in the right cerebellar hemisphere and pons (a). Brain CT showed large left thalamic hematoma with rapture into the lateral ventricle (b). (c, d) Patients 2. Fluid-attenuated inversion recovery (FLAIR) MRI showed multiple small-vessel occlusions accompanied by marked leukoaraiosis (c). Magnetic resonance angiography (MRA) showed moderate dolichoectasia of the basilar and vertebral arteries (d). (e, f) Patient 3. T2- (e) and T2*-weighted (f) MRI showed multiple small-vessel occlusions and microbleeds in the cerebral white matter and basal ganglia. Microbleeds were also observed in the dentate nucleus. (g, h) FLAIR MRI of patient 4 (g) and patient 5 (h) showed multiple small-vessel occlusions in the cerebral white matter. Leukoaraiosis was also observed in patient 4 (g).

(Fig. 3c,e,g). Intracerebral hemorrhage was observed in four patients. Three patients had a history of symptomatic intracerebral hemorrhage (Fig. 3b) and the other patient had asymptomatic multiple microbleeds detected by T2*-weighted MRI (Fig. 3f). Two patients took low doses of aspirin when they developed cerebral hemorrhage. Vertebrobasilar dolichoectasia was observed in one patient (Fig. 3d). Increased signal intensity in the pulvinar region on T1-weighted MRI was not observed in any patients. Two patients had a history of hypertension; however, their blood pressures were well controlled by antihypertensive drugs. None of the patients had other common risk factors for stroke, including dyslipidemia, diabetes mellitus, hyperuricemia, and smoking. No patients with the *GLA* p.E66Q mutation showed characteristic symptoms of Fabry disease, such as renal dysfunction, cardiomyopathy, acroparesthesia, hypohidrosis, and angiokeratoma. None of the patients with the p.E66Q mutation had a family history of Fabry disease, although an uncle of patient 1 and mothers of patients 2 and 3 had histories of cerebral infarction, and a brother of patient 2 had a history of chronic renal failure (Figure S1).

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Allele frequencies of the *GLA* p.E66Q mutation in the Japanese population and statistical analysis

To estimate the frequency in the general Japanese population of the *GLA* p.E66Q mutation found in the IS patient cohort, the data from newborn screening for Fabry disease performed in the Kumamoto prefecture from October 2009 to May 2010 were used. In this screening, 5051 consecutive male neonates (5051 alleles) were tested. This study was approved by the Kumamoto University Ethics Committee and written informed consent was obtained from each parent prior to enrollment. Genomic DNAs were isolated from whole blood, and exon 2 of the *GLA* gene was amplified by PCR. The amplification product was analyzed by direct sequencing. Thirty-two hemizygous male neonates were identified, and the allele frequency of the *GLA* p.E66Q in the Japanese population was thus determined to be 0.637%. Statistical analysis using Fisher's exact test indicated that the allele frequency of *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (OR = 3.34, $P = 0.025$; Table 3). However, in all IS, large-artery atherosclerosis, cardioembolism, and non-cardioembolism patients, the ORs were 1.67, 1.39, 0, and 2.01, respectively; the differences were not statistically significant (Table 3).

Discussion

Screening for Fabry disease in high-risk populations became an important concern when enzyme replacement therapy became available [25]. Studies performed in different settings indicated severe complications of Fabry disease, including left ventricular hypertrophy/hypertrophic cardiomyopathy [8,13,14], renal insufficiency [7,11,12], and stroke [15–21]. The prevalence of unrecognized Fabry disease in young male patients with stroke was first reported in 2005 [15]. Since then, several studies have estimated the prevalence of Fabry disease in young male patients with stroke as ranging from 0% to 4.9% [15–20]. Recently, Rolfs *et al.* [19] reported the results of the largest screening for Fabry disease in young patients with acute cerebrovascular disease. They enrolled 5023 patients from 15 European countries and found 27 patients (0.54%) with definite Fabry disease and 18 patients (0.36%) with probable Fabry disease. However, most stroke patients are elderly and there has been only one population-based study in unselected patients with stroke [21].

In the present study, five patients were identified as having *GLA* mutation and all of them had the same missense mutation, c.196G>C (p.E66Q). All patients with the p.E66Q mutation showed a similar clinical picture, i.e. multiple small-vessel occlusions with a high frequency of intracerebral hemorrhage. Interestingly, most patients with this mutation lacked common risk factors for stroke. Only two patients had hypertension and their blood pressures were well controlled by antihypertensive drugs (Table 2). The p.E66Q mutation was first identified in a male patient with classic-type Fabry disease. However, he also had another *GLA* missense mutation, p.R112C, in the same allele, which was predicted to cause a large structural change in the α-Gal A protein that leads to classic-type Fabry disease [26]. Subsequently, 26 patients with *GLA* p.E66Q mutation who developed adult-onset left ventricular hypertrophy or renal insufficiency were identified [7,12,27–29], and this mutation was therefore considered to be pathogenic, causing late-onset variant Fabry disease. However, none of these studies provided histological evidence confirming the diagnosis of Fabry disease in such cases. Recently, subjects harboring the p.E66Q mutation in the *GLA* gene have been found at unexpectedly high frequencies amongst Korean [30] and Japanese [31] populations, which has raised interest in the possibility that p.E66Q is a disease-causing mutation or a functional polymorphism.

Table 3 Allele frequencies of the *GLA* p.E66Q mutation in the Japanese men

	Control (newborn screening)[a]	All IS patients[b]	Subtypes of IS			
			Large-artery atherosclerosis[b]	Cardioembolism[b]	Small-vessel occlusion[b]	Non-cardioembolism[b]
Number of subjects	5051	475	114	79	240	396
Number of subjects with p.E66Q	32	5	1	0	5	5
p.E66Q allele frequency (%)	0.64	1.05	0.88	0	2.08	1.41
Odds ratio (versus control)		1.67	1.39	0	3.34	2.01
P-value (versus control)		0.244	0.522	1	0.025	0.188

[a]Frequency of p.E66Q mutation was determined by DNA sequencing;
[b]frequency of p.E66Q mutation was determined by enzymatic screening followed by DNA sequencing.

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Recent studies have shown that the structure of the α-Gal A protein with the p.E66Q amino acid substitution was less stable than that of the wild-type protein [31], and plasma and white blood cell α-Gal A activities in male subjects harboring the p.E66Q mutation were 13–26% and 19–65% of the normal mean α-Gal A activity, respectively [30,31], compatible with our dried blood spot enzymatic assay results (*c.* 40% of the normal mean; Fig. 2). The levels of these residual α-Gal A activities of the individuals with p.E66Q mutation were almost the same as those of male stroke patients with p.R118C and p.D313Y mutations identified in a Portuguese screen (PORTYSTROKE study) [17]. *GLA* p.D313Y mutation was found in 0.45% of normal X chromosomes in the Caucasian population [32]. In the PORTYSTROKE study [17], the allele frequency of p.D313Y in the stroke population was higher than that in normal controls, although the difference was not statistically significant. In this study, it was shown that the allele frequency of the *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (OR = 3.34, $P = 0.025$; Table 3), indicating that this mutation confers a high risk of small-vessel occlusion in Japanese males.

In this study, the frequency of the p.E66Q mutation in control subjects was determined by DNA sequencing, whilst that in stroke patients was determined by enzymatic screening followed by DNA sequencing. Therefore, the frequency of p.E66Q in stroke patients is likely to have been underestimated. Fujii *et al.* [29] analyzed the prevalence of Fabry disease in Japanese hemodialysis patients using dried blood spot screening followed by DNA sequencing, which was identical to the method used in the present study. They found only one patient with the p.E66Q mutation amongst 625 Japanese male hemodialysis patients (the prevalence of the p.E66Q mutation was 0.16%), which was much lower than that in our newborn DNA screen (Table 3), suggesting that a substantial number of patients with this mutation may have been missed in dried blood spot enzymatic screening. In addition, eight patients whose α-Gal A activities were below the cut-off value at the initial screening could not be followed up, because they moved to other hospitals or clinics. Therefore, there may have been additional patients with *GLA* mutations amongst those who dropped out, and the frequency of p.E66Q in stroke patients might thus have been underestimated in this study. Another point to be taken into consideration is patient selection bias, as patients were enrolled only from selected neurology departments, whilst a substantial number of stroke patients may be managed by neurosurgeons. In addition, informed consent could not be obtained from some of the severe stroke patients. These may explain why the proportion of small-vessel occlusion was more than 50% in this study. Although DNA sequencing of the *GLA* gene in all patients is necessary to determine the precise frequency of p.E66Q mutation in stroke patients, it is clear that p.E66Q mutation in the *GLA* gene is an important genetic risk factor for small-vessel occlusion in elderly Japanese males.

The precise pathomechanism by which *GLA* p.E66Q mutation increases the risk of lacunar infarction remains unknown. Recently, it was reported that cerebral small-vessel disease, which is known to be associated with lacunar infarction, white matter lesions (leukoaraiosis), and cerebral hemorrhage, rather than large-artery stroke, is frequently observed in Fabry disease [4,5,33,34]. These observations are compatible with clinical findings of our patients with p.E66Q mutation who developed multiple small-vessel occlusions and cerebral hemorrhage. Our findings suggest that *GLA* mutations associated with relatively high residual α-Gal A activity may add to the risk of cerebral small-vessel disease, possibly by contributing to the underlying multifactorial pathogenesis rather than through a classic Mendelian effect.

Enzyme replacement therapy (ERT) is currently the only approved therapy for Fabry disease. However, patients with *GLA* p.E66Q are not considered to be candidates for ERT, as p.E66Q is not a causative mutation for classic-type or variant-type Fabry disease [30,31]. On the other hand, Shimotori *et al.* [28] reported that 1-deoxygalactonojirimycin, an active site-specific pharmacological chaperone (ASSC), significantly increased α-Gal A activity of COS-7 cells with *GLA* p.E66Q mutation, suggesting that ASSC may be a potential therapeutic option for patients with this mutation.

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Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pedigrees of patients with p.E66Q mutation.

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